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PCT/FI2004/000204

Helsinki 12.5.2004

E T U O I K E U S T O D I S T U S
P R I O R I T Y D O C U M E N T

REC'D 27 MAY 2004
WIPO PCT

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Patenttihakemus nro
Patent application no

20030615

Tekemispäivä
Filing date

24.04.2003

Kansainvälinen luokka
International class

C12N

Keksinnön nimitys
Title of invention

"Nanoparticle for bioaffinity assays"
(Nanopartikkeli bioaffiniteettimäärityksiin)

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NANOPARTICLE FOR BIOAFFINITY ASSAYS

FIELD OF INVENTION

This invention relates to nanoparticles for bioaffinity assays.

BACKGROUND OF THE INVENTION

The publications and other materials used herein to illuminate the background of the invention and, in particular, cases to provide additional details respecting the practice, are incorporated by reference.

Expression of foreign proteins or peptides on the surface of viruses as virus surface protein fusions has been widely studied. The most common purpose of this research has been the use of viruses as tools for biopanning technology used in protein or peptide engineering. In these studies, the most widely used virus has been the *Escherichia coli* filamentous bacteriophage M13 and it has been considered the most suitable for protein or peptide display (US5,223,409; US5,403,484; US5,969,108 and US5,885,793). The display of proteins and peptides on the surface of bacteriophages T4, T7 and λ have also been studied [Sternberg, N. & Hoess, R. (1995) Display of peptides and proteins on the surface of bacteriophage λ, *Proc. Natl. Acad. Sci. USA* **92**, 1609-1613; Ren, Z. & Black, L. (1998) Phage T4 SOC and HOC display of biologically active, full-length proteins on the viral capsid, *Gene* **215**, 439-444; Danner, S. & Belasco, J. (2001) T7 phage display: A novel genetic selection system for cloning RNA-binding proteins from cDNA libraries, *Proc. Natl. Acad. Sci. USA* **98**, 12954-12959] for biopanning purposes. In these examples phages connect the binding activity located on the suface of particle to genetic information located inside the particle.

There has also been research on the expression of foreign proteins on the surface of viruses with the goal of using these modified viruses as vaccines, immunogens and coating agents (US5,008,373; US5,041,385; US5,463,024; US5,736,368; US5,804,196 and US6,051,410). All these applications have in common the fact that the viral surface is used as a passive solid state support only, without any functionality of the capsid in addition to the holding together of the displayed proteins or peptides spatially.

Research on *Escherichia coli* bacteriophage T4 has shown that the three internal proteins IPI, IPII and IPIII are targeted inside the phage capsid by a signal-peptide dependent transport mechanism. This signal peptide known as capsid targeting sequence (CTS, sequence: Met-Lys-Thr-Tyr-Gln-Glu-Phe-Ile-Ala-Glu) can also target foreign proteins inside the capsid, if it is cloned in the front of these proteins. At least *Staphylococcus aureus* endonuclease (SN), green fluorescent protein (GFP), *EcoRI* restriction endonuclease, β-galactosidase and an inactive firefly lusiferase fragment have been successfully targeted inside T4 phage capsid, but of these only SN and GFP demonstrated functionality inside the capsid [Mullaney, J. & Black, L. (1998) Activity of foreign proteins targeted within the bacteriophage T4 head and prohead: Implications for packaged DNA structure, *J. Mol. Biol.* **283**, 913-929; Mullaney, J. & Black, L. (1996) Capsid targeting sequence targets foreign proteins into bacteriophage T4 and permits proteolytic processing, *J. Mol. Biol.* **261**, 372-385]. The reason for the inactivity of the rest of the tested proteins was claimed to be the multimeric nature of these proteins, the absence of appropriate substrate inside the phage capsid and/or the inability of large substrate or cofactor molecules (>3.5 nm in diameter) to penetrate the phage capsid.

A search of the sequence databanks for the sequence of the CTS peptide reveals that at least bacteriophage K3, RB70 and RB15 have genes with the CTS sequence in them. This could mean that these phages also possess a similar capsid targeting machinery as phage T4.

Viruses have been shown to be self-assembling. The different proteins expressed from the viral genome hold all the necessary information for the formation of the viral particle. In many cases, several proteins and/or the viral DNA can be omitted from the component pool and the virus still manages to self-assemble (the resulting particles of course lack the omitted components and may be thus distorted with respect to the normal viruses). In some cases parts of the virus such as the head can self-assemble when just a few crucial proteins are produced separately and then combined *in vitro* [Cerritelli, M. & Studier, W. (1996) Assembly of T7 capsids from independently expressed and purified head protein and scaffolding protein. *J. Mol. Biol.* **258**, 286-298].

A marker is a molecule, which is possible to detect by chemical or physical means. Marker may have catalytic activity which is used for the detection. Examples of those markers are nucleic acids or proteins that have catalytic activity. A useful way to detect a marker is based on its fluorescence, luminescence, optical, electric or magnetic properties. Marker proteins are widely used in biological research and especially in bioaffinity assays. The following enzymes are examples of widely used marker proteins: alkaline phosphatase (EC 3.1.3.1), ß-Galactosidase (EC 3.2.1.23), ß-Glucuronidase (EC 3.2.1.31), glucose oxidase (EC 1.1.3.4), luciferase (EC 1.13.12.7) and horseradish peroxidase (EC 1.11.1.7). The common feature with all of them is that their detection is possible at low concentrations by using a simple protocol. In addition to enzymes, alternative marker proteins have been described, such as fluorescent proteins [Heim R, and Tsien RY, (1996), Current Biology 6: 178-82] or colored proteins [Lukyanov KA, Fradkov AF, et. al. (2000), Journal of Biological Chemistry 275: 25879-82].

Numerous well-defined conjugates between a binding molecule and a marker protein for assay development have been produced. A conjugate is traditionally produced by *in vitro* labeling of binding molecule with marker protein or peptide [Kopetzki, E.; Lehnert, K; Buckel, P. Clin. Chem. (1994), 40: 688-704]. An alternative way to produce the conjugate is to fuse genes encoding a binding

molecule and a reporter protein, which results in the production of a fusion protein having both binding and marker activity [Zenno and Inouye, Biochemical and Biophysical Research Communications (1990), 171:169-74].

OBJECT AND SUMMARY OF THE INVENTION

One object of the present invention is to provide improved particles for bioaffinity assays.

Another object of the present invention is to provide improved bioaffinity assays making use of the improved particles.

Yet another object of the present invention is to provide improved kits for bioaffinity assays making use of the improved particles.

Thus this invention provides a nanoparticle, useful for bioaffinity assays, comprising a self-assembling shell built up of several protein and/or peptide subunits, which protein and/or peptide subunits can be of one or several different types, assembled in an organized manner to form the shell having an inner surface facing the inside and an outer surface facing the outside of said particle wherein

a) one or several of the types of subunits have one or several first binding moieties per type of subunit with the binding moiety facing the outside of the particle for binding of any specific ligand binding protein; and

b) i) the particle contains within its shell a marker and/or

ii) one or several of the types of subunits have one or several second binding moieties per type of subunit with the binding moiety facing the inside and/or the outside of the particle for binding a marker; and

c) the marker or markers enables detection of the particle.

This invention also provides a bioaffinity assay using the nanoparticle.

This invention further provides a kit for a bioaffinity assay comprising the nanoparticle.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the structure of a protein nanoparticle.

Figure 2 shows detection of the binding activity of a protein particle.

Figure 3 shows the structure of plasmid pPrGHoc.

Figure 4 shows the structure of plasmid pTSHscHoc

Figure 5 shows detection of anti-TSH activity on the surface of phage based particles.

Figure 6 shows the structure of plasmid pCBPHoc.

Figure 7 shows the structure of plasmid pBCCPHoc.

Figure 8 shows the structure of plasmid pPrGHFl.

Figure 9 shows the structure of plasmid pTSHscHFl.

Figure 10 shows the structure of plasmid pCBPHFl.

Figure 11 shows the structure of plasmid pCTSGalO.

Figure 12 shows detection of galactose oxidase activity inside a phage based particle.

Figure 13 shows the structure of plasmid pCTSluc.

Figure 14 shows the detection of luciferase activity inside a phage based particle.

Figure 15 shows the structure of plasmid p602/TSHscHoc.

Figure 16A and 16B show the use of a nanoparticle in an immunoassay. Figure 16A shows the principle of the assay and Figure 16B a standard curve of the assay.

DETAILED DESCRIPTION OF THE INVENTION

Definition of terms

The term "bioaffinity assays" shall be understood to include one-step and multi-step competitive and non-competitive ligand binding assays and immunoassays based on a single or multiple specific ligand binding moieties, e.g. monoclonal antibodies, polypeptides, receptors, recombinant antibodies or antibody fragments as well as artificial binders like aptamers and engineered proteins.

Bioaffinity assays include heterogenous and homogenous assays. In heterogenous assay the analyte is bound to a solid phase and particle is used for the detection of bound analyte. A particle can bind directly to the analyte or to a molecule which is bound to the analyte. Particle and analyte can be added to reaction either sequentially or simultaneously. In homogenous assay the analyte is detected from solution without the separation of unbound particle. Homogenous assay can be based on for example Fluorescence Resonance Energy Transfer (FRET) or Bioluminescence Resonance Energy Transfer (BRET) (Boute, N, Jockers, R and Issad, T. 2002. *Trends in Pharmacological Sciences* 23:351-354). It can also be based on channeling of substrate, product or intermediate of an enzyme reaction (Gibbons, I, Armenta, R, DiNello, RK and Ullman, EF. 1987. *Methods in Enzymology* 136:93-103).

The term "marker" shall be understood as a feature detectable by measuring luminescence or absorbance, as well as other optical properties, electrical properties e.g. electrical current or voltage or magnetic properties, originating directly or resulted indirectly from the existence of the feature. Example of direct measurement

of the feature is measurement of fluorescence emission of green fluorescent protein using appropriate excitation light. Example of indirect measurement of the feature is measurement of luminescence originating from a chemical reaction catalysed by luciferase enzyme marker. Example of measurement of electrical properties is e.g. detection of redox-reaction by measuring electrical voltage or current.

The term "luminescence" shall be understood to cover luminescence, bioluminescence, chemiluminescence, electroluminescence, photoluminescence, fluorescence, delayed fluorescence and phosphorescence.

The term "luminescent protein" and "fluorescent protein", respectively, shall be understood as a protein or enzyme, which produces luminescence or is fluorescent, respectively, with or without prosthetic groups. Example of luminescent protein is luciferase enzyme. Example of fluorescent protein is green fluorescent protein (GFP).

The term "nanoparticle" shall be understood as a particulate reagent composed of multiple "subunits", each composed of proteins or polypeptides and, in addition, optionally of single or multiple features of the following: nucleic acids, prosthetic groups, organic and inorganic compounds. The particulate has at least a single "binding moiety"on the outer surface and the particulate contains a single "marker" or multiple "markers". Dimensions of the particulate are between one nanometer and ten micrometers.

The term "lanthanide" and "rare earth metal" shall be understood to include elements and combinations of different elements of rare earth ions from the following: europium (Eu), samarium (Sm), terbium (Tb), dysprosium (Dy), gadolinium (Gd), ytterbium (Yb), yttrium (Y) and neodymium (Nd).

The term "subunit" shall be understood as a single protein or polypeptide or complex of multiple proteins or polypeptides, composed of identical or different components.

The term "binding moiety" shall be understood to cover monoclonal antibodies, polypeptides, receptors, recombinant antibodies or antibody fragments as well as artificial binders like aptamers and engineered proteins, or derivatized form of any of the listed features. Example of polypeptide is calmodulin binding peptide (CBP). Example of derivatized feature is a peptide sequence of biotin carboxyl carrier protein (BCCP), which can be biotinylated in vivo with BirA biotin ligase enzyme.

The term "enzyme" shall be understood a protein or polypeptide or nucleic acid with catalytical activity. Examples of enzymes are lusiferase and galactose oxidase (GAO).

The term "galactose oxidase (GAO)" shall be understood as enzyme with Enzyme Commision number EC 1.1.3.9.

The term "colored protein" shall be understood as a protein or polypeptide, which has a significant absorption at visible wavelengths, 300 – 700 nm, with or without prosthetic groups.

The term "organic molecule" shall be understood as any chemical compound containing at least carbon with molecular weight below 7000 Dalton. Examples of organic molecules are prosthetic groups in fluorescent allophycocyanin protein.

The term "inorganic molecule" shall be understood as any inorganic atom, chemical compound composed of inorganic atoms or combination of atoms in an organized manner. Example of inorganic molecule is fluorescent CdSe semiconductor particle.

The term "self-assembling shell" shall be understood as a particulate structure capable of assembling itself from a pool of vital capsid proteins.

The term "virus capsid" shall be understood as the protein shell protecting viral DNA.

The term "vital capsid protein" shall be understood as protein which is needed for the self-assembly of a particulate entity e.g. nanoparticle.

The term "GFP" shall be understood as green fuorescent protein from *Aequorea victoria*, its mutant derivatives or homologous protein from other species.

The term "CRP" shall be understood as C-reactive protein.

The term "TSH" shall be understood as thyroid stimulating hormone.

The term "BCCP" shall be understood as Biotin carboxyl carrier protein.

The term "Protein G" shall be understood as Protein G from bacteria in genera *Streptococcus.*

The term "Protein A" shall be understood as Protein A from bacteria in genera *Staphylococcus.*

The term "Protein L" shall be understood as Protein L from bacteria in genera *Peptostreptococcus.*

General description of preferred embodiments

The nanoparticles according to the invention can have useful properties. These can be, but are not limited to, low cost, simple production, high stability and highly defined structure. Production can be very simple whereas a simple microbial fermentation with minor down-stream processing is typically all that is needed.

The nanoparticle according to the invention can have a marker that is an enzyme, luminescent protein, fluorescent or coloured protein or organic molecule, or a rare earth metal. If the marker is a protein, it can be an enzyme such as luciferase or GAO, or a fluorescent protein like GFP. If the marker is a rare earth metal ion, it can be a Tb, Eu, Sm or Dy ion.

The nanoparticle of the invention can have, in addition to the first and second binding moieties third binding moieties. One or several of the types of subunits can e.g. have one or several third binding moieties per type of subunit with the binding moiety facing the outside of the particle for binding to a solid support. The nanoparticle can also have additional binding moieties with additional functions.

The shell of the nanoparticle can be a recombinant virus capsid or a self-assembling shell made of vital capsid protein(s). The virus can be a phage, preferably a T4, K3, RB70 or RB15 phage. The shell of the nanoparticle can also be a recombinant apoferritin.

The first, second, third or additional binding moiety can be protein A, protein G, protein L or calmodulin binding peptide (CBP). The first, second, third or additional binding moiety can be an antibody against e.g. CRP, ABO blood group antigens or TSH. The first, second, third or additional binding moiety can be protein A, protein G, protein L or CBP.

The minimum radius of the nanoparticle is typically more than 10 nm, preferably more than 40 nm, even more preferably more than 60 nm. The number of subunits of the shell of the nanoparticle is typically more than 8, preferably more than 20, more preferably more than 400, most preferably more than 900.

Description of the figures

Figure 1 shows the structure of a protein nanoparticle according to the invention. The figure shows a protein shell 1, a first binding molecule 2 facing the outside of the nanoparticle, a marker 3 within the shell of the nanoparticle and a second or third binding molecule 4 facing the outside of the nanoparticle.

Figure 2 shows detection of binding activity of a protein particle. Analyte specific to the binding molecule to be tested is labelled with a molecule suitable for detection. Labelled analyte is then reacted with particles and particle/analyte

complexes are separated from unbound analyte by gel filtration and the signal of the label is measured.

Figure 3 shows the structure of plasmid pPrGHoc. Abbreviations stand for: ORI = Origin of replication, Lac PO = lac promoter/operator, Pamp = Promoter for beta-lactamase gene, amp = beta-lactamase gene, Protein GHoc = gene encoding Protein G-Hoc fusion protein.

Figure 4 shows the structure of plasmid pTSHscHoc. Abbreviations stand for: ORI = Origin of replication, Lac PO = lac promoter/operator, Pamp = Promoter for beta-lactamase gene, amp = beta-lactamase gene, LacI = gene encoding Lac repressor, TSHscHoc = gene encoding anti-TSHscFv-Hoc fusion protein.

Figure 5 shows detection of anti-TSH activity on the surface of phage based particles. The symbols stand for: triangle = particled with anti-TSHscFv on the surface, square = control particles without anti-TSHscFv.

Figure 6 shows the structure of plasmid pCBPHoc. Abbreviations stand for: ORI = Origin of replication, Lac PO = lac promoter/operator, Pamp = Promoter for beta-lactamase gene, amp = beta-lactamase gene, LacI = gene encoding Lac repressor, CBP-Hoc = gene encoding CBP-Hoc fusion protein.

Figure 7 shows the structure of plasmid pBCCPHoc. Abbreviations stand for: ORI = Origin of replication, Lac PO = lac promoter/operator, Pamp = Promoter for beta-lactamase gene, amp = beta-lactamase gene, LacI = gene encoding Lac repressor, Bccp-Hoc = gene encoding Bccp-Hoc fusion protein.

Figure 8 shows the structure of plasmid pPrGHFl. Abbreviations stand for: pBR322 ori = Origin of replication, Kan = kanamycin resistance gene, LacI = gene encoding Lac repressor, Protein G-ferritin = gene encoding Protein G-Human ferritin light chain fusion protein.

Figure 9 shows the structure of plasmid pTSHscHFl. Abbreviations stand for: anti-TSHsc-ferritin = gene encoding antiTSHscFv antibody-Human ferritin light chain fusion protein.

Figure 10 shows the structure of plasmid pCBPHFl. Abbreviations stand for: CBP-ferritin = gene encoding CBP-Human ferritin light chain fusion protein.

Figure 11 shows the structure of plasmid pCTSGalO. Abbreviations stand for: ORI = Origin of replication, Lac PO = lac promoter/operator, Pamp = Promoter for beta-lactamase gene, amp = beta-lactamase gene, CTSGalO = gene encoding Capsid targeting sequence fused with Galactose Oxidase.

Figure 12 shows detection of galactose oxidase activity inside phage based particle. The fractions of gel filtration experiment described in Example are analysed for the luminescence, which is produced in the oxidation of luminol by H_2O_2 produced by galactose oxidase.

Figure 13 shows the structure of plasmid pCTSluc. Abbreviations stand for: ORI = Origin of replication, Lac PO = lac promoter/operator, Pamp = Promoter for beta-lactamase gene, amp = beta-lactamase gene, CTSluc = gene encoding Capsid targeting sequence fused with luciferase.

Figure 14 shows detection of luciferase activity inside phage based particle. The fractions of gel filtration experiment described in Example 2 are analysed for the luminescence, which is produced in oxidation of firefly luciferase substrate D-luciferin by luciferase enzyme.

Figure 15 shows the structure of plasmid p602/TSHscHoc. Abbreviations stand for: ORI- = Origin of replication, lac/T5 = lac operator T5 promoter, cat = chloramphenicol acetyltrasferase gene, LacI = gene encoding Lac repressor, TSHscHoc = gene encoding anti-TSHscFv-Hoc fusion protein.

Figure 16A and 16B show the use of protein nanoparticles in an immunoassay. Figure 16A shows a streptavidin-coated solid support 5, biotinylated anti-TSH antibody 6, TSH 7 and protein nanoparticle 8 according to Example 17. Figure 16B shows the standard curve of the TSH immunoassay with the protein particles.

Methods

DNA manipulations

All DNA manipulations were made according to known protocols [Sambrook J, Fritsch EF, and Maniatis T, (1989), Molecular Cloning: A laboratory manual, Cold Spring Harbour Laboratory Press, Cold Spring Harbour),

Bacteria and phages strains

The following bacterial strains are used in the examples: XL-1 Blue, BL21, BL21(DE3), BL21(DE3:pLysS), Origami B, Origami B(DE3) and Origami B(DE3:pLysS) (Stratagene, La Jolla, CA, USA). T4 bacteriophages were Δ*hoc*- and Δ*soc*- mutants (Malys N, Chang DY, Baumann RG, Xie D, Black LW 2002. Journal of Molecular Biology 319:289-304).

Measurement of fluorescence

Time-resolved fluorescence of europium and terbium was measured with Delfia reagents from PerkinElmer Life Sciences (Boston, MA, USA). Fluoresece of Alexa Fluor 594 (Molecular Probes Europe, Leiden, The Netherlands) was measured as suggested by the manufacturer. All measurements were done with Wallac Victor multilabel counter (Perkin Elmer Life Sciences, Boston, MA, USA).

Detection of reporter proteins

The activity of firefly luciferase was determined by measuring luminescence produced with Luciferase Assay Kit from BioThema ltd (Haninge, Sweden).

The activity of galactose oxidase was determined by measuring luminescence from luminol oxidation by H_2O_2 generated in the reaction of enzyme with 50 mM galactose in 100 mM phosphate buffer containing 1 mM luminol and 0.4 mM $CuSO_4$ at pH 8.6. All measurements were done with Wallac Victor multilabel counter (Perkin Elmer Life Sciences, Boston, MA, USA).

Example 1

Production of recombinant phage based particles

Escherichia coli cells expressing plasmids encoding binding molecules and/or reporter molecules (Described in Example 6, Example 7, Example 8, Example 9, Example 15, Example 16 and Example 17) were grown in SB medium (30 g tryptone, 20 g yeast extract, 10 g MOPS, 1 g glucose, 2 g maltose, 10 mM $MgSO_4$ per liter, pH 7.0) in shaking at 37 °C until OD_{600} reached 0.2. The protein production was then induced by adding IPTG to the concentration of 0.5 mM and the culture was infected with T4 phages by using $2x10^8$ phages/ml. The cultivation was continued at 26 °C until the cells autolysed. Cell debris was removed by centrifugation at 5000*g* for 10 minutes. Phage particles were then pelleted by centrifugation at 13 000*g* for 50 minutes after which they were dissolved to buffer containing 5 mM Tris-HCl, 0.01% Tween-20 and 0.05% NaN_3 at pH 7.5.

Example 2

Purification of the particles with gel filtration

Protein nanoparticles were purified by gel filtration with 10 ml Sepharose 6B column (Amersham Biosciences Corp, Piscataway, NJ, USA). The column was first equilibrated by using buffer containing 5 mM Tris-HCl, 0.01% Tween-20 and 0.05% NaN_3 at pH 7.5 with 10 volumes of the column. A 500 µl sample was applied to the column and 1 ml fractions were collected. Protein nanoparticles eluted from the column in the fractions number 4-8 as shown in Figure 5 for phage based particles and 3-8 for ferritin based particles.

Example 3

Detection of binding activity on the surface of protein particle by using gel filtration

Rationale of the analysis of the activity of binding molecules on the surfaces of the particles is shown in Figure 2. Analyte specific to the binding molecule to be tested was labeled with a molecule suitable for detection. Analytes were labeled as follows: TSH (thyroid stimulating hormone) (Scripps Laboratories, San Diego, CA, USA), streptavidin (Perkin Elmer Life Sciences, Boston, MA, USA) and antibodies with Europium and Calmodulin with Alexa Fluor 594. Mixture of four monoclonal Eu-labelled antibodies was used in the analysis of Protein G activity. Labeling of the molecules with Europium was done with a reagent kit obtained from Perkin Elmer Life Sciences, (Boston, MA, USA). Labeled calmodulin was obtained from Molecular Probes Leiden, The Netherlands). Labeled analyte was reacted with particles and particle/analyte complexes were separated from unbound analyte by gel filtration as described in Example 2. Phage based particles were produced as described in Example 1. Ferritin based particles were produced as described in

Example 10. Particles produced without binding molecule were used as negative control.

Example 4

Detection of binding activity on the surface of protein particle by using multiwell plates

In vitro biotinylation of TSH and antibodies was done by reagent kit obtained from Perkin Elmer Life Sciences, Boston, MA, USA). Mixture of four monoclonal Eu-labelled antibodies was used in the analysis of Protein G activity. Biotinylated molecules were attached to streptavidin-coated 96 well plates (Innotrac Diagnostics, Turku, Finland) as described in the kit and wells were washed four times. After that the particles to be tested were added and the wells were again washed for four times. Then the analyte labelled as described in Example 3 was added, wells washed four times and signal from the label measured.

Example 5

Detection of binding activity on the surface of protein particle by using molecular weight cut-off filters

Analyte molecules were labelled as described in Example 3. Particles together with bound analyte were separated from smaller molecules with 100 kDa cut-off value filters (Pall Life Science, Ann Arbor, MI, USA). Signal of the labelled molecule was measured from the retentate.

Example 6

Production of Protein G on the surface of T4 phage

Gene encoding Hoc protein of T4 bacteriophage was amplified with polymerase chain reaction (PCR) with primers 5'-TACAAGTCCGGAAGCGGCCAT-ATGACTTTTACAGTTGATATAACTCCTAAAACACCTAC and 5'-GACGTC-GAATTCTTATGGATAGGTATAGATGATACCAGTTTCTAAAGCAG with T4 genomic DNA as template. The PCR product was digested with *Eco*RI and *Xba*I and ligated with *Aat*II and *Acc*III digested pEl19D (Maksimow M, Hakkila K, Karp M, and Virta M. 2002. Cytometry 43: 243-247.) The resulting construct was transformed to *E. coli* XL-1 Blue cells. The plasmid was isolated and its sequence verified by DNA sequencing. Gene encoding *Streptococcal* Protein G was amplified by using the primers 5'-GGAGATATACCCATGGTGACAACTTACAAAC and 5'-CATCACCAGGTCCGGAAACCTCTGTAACCATTTCAGTTAC with *Streptococcus* G148 genomic DNA as template. It was inserted to the plasmid by ligating *Nco*I - *Acc*III digested plasmid with *Nco*I and *Acc*III digested PCR fragment. The resulting construct was transformed to *E. coli* BL21 cells. Structure of the resulting plasmid, pPrGHoc, was verified by partial sequencing. The structure of pPrGHoc is shown in Figure 3. Particles expressing Protein G on their surface were produced with *E. coli* BL21(pPrHoc) cells by using protocol described in Example 1. The activity of Protein G was demonstrated as described in Example 4 by using Eu labelled antibodies as label and biotinylated antibodies as catcher molecules. The particles with Protein G gave 1.3-fold signal as compared with particles produced without Protein G.

Example 7

Production of scFv fragment on the surface of T4 phage

Gene encoding Protein G in plasmid pPrHoc was replaced with gene encoding scFv fragment of anti-TSH-antibody by ligating *Nco*I and *Acc*III digested PCR product obtained by primers 5'-GGAAACAGCCATGGAAATTGTGCTCACCCAGTCT-CCTG and 5'-CTGCCGCTTCCGGACCCCGAGGCCGCAGAGAC with plasmid containing anti-TSHscFv antibody gene (Nelli Stromsten 2000. MSc thesis, University of Turku, Finland) as template with *Nco*I and *Acc*III digested pPrGHoc. The resulting construct was transformed to *E. coli* Origami B cells. Structure of the resulting plasmid, pTSHscHoc, was verified by partial sequencing. The structure of pTSHscHoc is shown in Figure 4. Particles expressing anti-TSHscFv antibody on their surface were produced with *E. coli* Origami B(pTSHscHoc) cells by using protocol described in Example 1. The activity of anti-TSH is shown in Figure 5 where fluorescence of fractions of an experiment described in Example 3 are shown.

Example 8

Production of Calmodulin Binding Peptide on the surface of T4 phage

Gene encodin cbp was constructed by self annealing oligonucleotides 5'-GAGATATACCATGGCGGCTGCACGTTGGAAAAAAGCGTTCATCGCTG TTTCTGCTGCAAACCG and 5'-TGGCCGCTTCCGGAGCCGCCGCTACCA-CCGCTGATTTTCTTGAAACGGTTTGCAGCAGAAACAGCG. The strands were completed with DNA polymerase treatment and the fragment digested with *Nco*I and *Acc*III. The digested fragment was ligated with *Nco*I-*Acc*III fragment of pTSHscHoc. The resulting construct was transformed to *E. coli* BL21 cells. Structure of the resulting plasmid, pCBPHoc, was verified by partial sequencing.

The structure of pCBPHoc is shown in Figure 6. Particles expressing cbp on their surface were produced with *E. coli* 21(pCBPHoc) cells by using protocol described in Example 1. The activity of cbp was demonstrated as described in Example 3 with Alexa Fluor 594 labelled calmodulin as label. The particles with cbp gave 8.1-fold signal as compared to particles without cbp.

Example 9

Production of Biotinylated Peptide on the surface of T4 phage

Gene encoding Protein G in plasmid pPrHoc was replaced with gene encoding Biotin Carboxyl Carrier Peptide (bccp) by ligating *Nco*I and *Acc*III digested PCR product obtained by primers 5'-AGGAGATATACCATGGGTCACATCGTA-CGTTCCCCG and 5'-TATGGCCGCTTCCGGACTCGATGACGACCAGCGGC with *E. coli* XL-1 Blue genomic DNA as template with *Nco*I and *Acc*III digested pPrGHoc. The resulting construct was transformed to *E. coli* BL21 cells. Structure of the resulting plasmid, pBCCPHoc, was verified by partial sequencing. The structure of pBCCPHoc is shown in Figure 7. Particles expressing bccp on their surface were produced with *E. coli* BL21(pBCCPHoc) cells by using protocol described in Example 1. The activity of bccp was demonstrated as described in Example 4 by using Eu labelled streptavidin as label. The particles with bccp gave 12-fold signal as compared with particles produced without bccp.

Example 10

Production of ferritin based particles

Escherichia coli cells expressing plasmids encoding a binding molecule fused to N-terminus of ferritin (Example 12, Example 13 and Example 14) subunits were grown in 50 ml of SB medium in shaking at 37 °C until OD_{600} reached 0.4. The

protein production was induced by adding IPTG to the concentration of 0.5 mM and the cultivation was continued at 26 °C over night. After that the cells were collected by centrifugation at 1500*g* for 10 minutes and suspended to 5 ml of phosphate-buffered saline (PBS). The cells were then lysed by sonication and cell debris removed by centrifugation at 5000*g* for 10 minutes. Supernatant was then filtered with 100 kDa cut-off value filters (Pall Life Science, Ann Arbor, MI, USA) and retentate was suspended to PBS.

Example 11

Loading of ferritin based particles with lantanides

The reaction buffer used in the loading of terbium ions into ferritin consisted of 50 mM HEPES, 50 mM NaCl and 10 mM $TbCl_3$ at pH 7.0. Ferritin was added to the buffer to 0.1 μM and the reaction was incubated at 37 °C for 20 h. Unreacted terbium was removed by gel filtration with NAP-5 column (Amersham Biosciences Corp, Piscataway, NJ, USA).

Example 12

Production of Protein G on the surface of ferritin

Gene encoding *Streptococcal* Protein G was inserted to plasmid producing human ferritin light chain by ligating *Nhe*I digested fragment obtained by PCR with oligonucleotides 5'-AAGGATCCCATATGAACCTCTGTAACCATTTCAG and 5'-AACCATGGCATATGGTGACAACTTACAAACT with *Streptococcus* G148 genomic DNA as template with *Nhe*I digested plasmid pET-26(+)rHuLFt (Grace JE Jr, Van Eden ME, Aust SD. 2000. Archives in Biochemistry and Biophysics 384:116-22). The resulting construct was transformed to *E. coli* BL21(DE3:pLysS) cells. Structure of the resulting plasmid, pPrGHFl, was verified by partial

sequencing. The structure of pPrGHFl is shown in Figure 8. Ferritin based particles expressing Protein G on their surface were produced with *E. coli* BL21(DE3:pLysS:pPrGHFl) cells by using protocol described in Example 10. The retentate was suspended to 1 ml and diluted tenfold prior to the analysis of the functionality according to Example 4 using Eu-labelled antibodies as label. The particles with Protein G gave 5.5 fold signal as compared to particles produced with pET-26(+)rHuLFt.

Example 13

Production of scFv fragment on the surface of ferritin

Gene encoding anti-TSHscFv fragment was inserted to plasmid producing human ferritin light chain by ligating *Nhe*I digested fragment obtained by PCR with oligonucleotides 5'-GTTATATCAACTGTAAAAGT and 5'-AACCATGGCATA-TGGAAATTGTGCTCACCCA with pTSHscHoc as template with *Nhe*I degested plasmid pET-26(+)rHuLFt (Grace JE Jr, Van Eden ME, Aust SD. 2000. Archives in Biochemistry Biophysics 384:116-22). The resulting construct was transformed to *E. coli* Origami B(DE3:pLysS) cells. Structure of the resulting plasmid, pTSHscHFl, was verified by partial sequencing. The structure of pTSHscHFl is shown in Figure 9. Ferritin based particles expressing anti-TSHscFv antibody on their surface were produced with *E. coli* Origami B(DE3:pLysS:pTSHHFl) cells by using protocol described in Example 10. The retentate was suspended to 1 ml and diluted fivefold prior to the analysis of the functionality according to Example 4 using Eu-labelled antibodies as label. The particles with anti-TSHscFv gave 33-fold signal as compared to particles produced with pET-26(+)rHuLFt.

Example 14

Production of Calmodulin Binding Peptide on the surface of ferritin

Gene encodin cbp was constructed in two PCR reactions. First PCR was done with oligonucleotides 5'-GAATTCGGATCCTTAGTCGTGCTTG and 5'-CTGCTG-CGAACCGTTTCAAGAAAATCAGCTCTTCCGGTGCTGGCGGTATGAGCT-CCCAGATTCGTCAGAATT with pET-26(+)rHuLFt as template. The product of the first PCR was the used as template of the second PCR with oligonucleotides 5'-GAATTCGGATCCTTAGTCGTGCTTG and 5'-TAGATATACATATGAAAC-GCCGTTGGAAGAAAGCGTTCATCGCTGTTTCTGCTGCGAACCGTTTCA-AGAAAAT. The *Nde*I-*Bam*HI digested second product was ligated with 5.3 kb *Nde*I-*Bam*HI fragment of pET-26(+)rHuLFt. The resulting construct was transformed to *E. coli* BL21(DE3:pLysS) cells. Structure of the resulting plasmid, pCBPHFl, was verified by partial sequencing. The structure of pCBPHFl is shown in Figure 10. Ferritin based particles expressing cbp on their surface were produced with *E. coli* BL21(DE3:pLysS:pCBPHFl) cells by using protocol described in Example 10. The retentate was suspended to 1 ml and diluted five-fold prior to the analysis of the functionality according to Example 3 with Alexa Fluor 594 labelled calmodulin. The particles with cbp gave 4.1-fold signal as compared to particles produced with pET-26(+)rHuLFt.

Example 15

Production of galactose oxidase inside T4 phage

Gene encoding galactose oxidase gene was amplified from plasmid pGAOA3.E7-036 (Sun L, Petrounia IP, Yagasaki M, Bandara G, Arnold FH 2001. Protein Engineering 14:699-704) by PCR with the following primers 5'-TATTGCCGA-AGCTTTGGCCTCAGCACCTATCGGAAGCGCC and 5'-CTAGAGTCGCGG-

CCGCTTACTGAGTAACGCGAATCGTCGAAGCC. The *Hind*III- *Not*I digested PCR product was ligated with 2.6 kb *Hind*III- *Not*I fragment of pEGFP (Clontech, Palo Alto, CA, USA) and the resulting construct was transformed to *E. coli* BL21 cells. Structure of the resulting plasmid, pCTSGalO, was verified by partial sequencing. The structure of pCTSGalO is shown in Figure 11. Particles expressing galactose oxidase inside their capsids were produced with *E. coli* BL21(pCTSGalO) cells by using protocol described in Example 1. The activity of galactose oxidase is demonstrated in Figure 12 where luminescence produced by oxidation of luminol by H_2O_2 produced by galactose oxidase in the fractions of gel filtration experiment described in Example 2 is shown.

Example 16

Production of luciferase inside T4 phage

Gene encoding luciferase from *Photinus Pyralis* as 1.7 kb *Not*I-*Pst*I fragment of pBluc (Bonin AL, Gossen M, and Bujard H. 1994. Gene 141:75-77) was used to replace galactose oxidase gene in pCTSGalO and the resulting construct was transformed to *E. coli* BL21 cells. Structure of the resulting plasmid, pCTSluc, was verified by partial sequencing. The structure of pCTSluc is shown in Figure 13. Particles expressing luciferase inside their capsides were produced with *E. coli* BL21(pCTSluc) cells by using protocol described in Example 1. The activity of luciferase is demonstrated in Figure 14 where luminescence produced by luciferase in the fractions of gel filtration experiment described in Example 2 is shown.

Example 17

Production of protein particles with scFv fragment on the surface and luciferase inside the particle

In order to produce protein nanoparticles having binding activity on the surface and marker protein activity inside, it was necessary to produce binding molecule and marker protein in same bacterial cell. For that purpose, plasmid 602/TSHscHoc which is capable in replicating in same cell with pCTSluc was constructed by ligating blunted 3.5 kb *Nsp*I-*Eco*RI fragment of pTSHscHoc with blunted 2.3 kb *Afl*II-*Kpn*I fragment of p602/22 (LeGrice S, Beuck V, and Mous J. 1987. Gene 55: 95-103.) and the resulting construct was transformed to *E. coli* Origami B cells. Structure of the resulting plasmid, p602/TSHscHoc, was verified by partial sequencing. The structure of p602/TSHscHoc is shown in Figure 15. *E. coli* Origami B(p602/TSHscHoc) cells were transformed with pCTSluc. The resulting strain, Origami B(p602/TSHscHoc:pCTSluc) was used for producing particles expressing luciferase inside and anti-TSH activity on the surface by using protocol described in Example 1. The particles were used to assay TSH as described in Example 18.

Example 18

Immunoassay of TSH with protein nanoparticles

The capture antibody was attached to multiwell plates as described in Example 4 and different amount of TSH was added to the wells. The wells were washed four times and tenfold diluted protein particles produced as described in Example 17 were added. The wells were washed four times and the activity of luciferase was measured as descibed in 'Detection of reporter proteins'. Luminescence signals are presented in Figure 16.

It will be appreciated that the present invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent for the specialist in the field that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

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CLAIMS

1. A nanoparticle, useful for bioaffinity assays, comprising a self-assembling shell built up of several protein and/or peptide subunits, which protein and/or peptide subunits can be of one or several different types, assembled in an organized manner to form the shell having an inner surface facing the inside and an outer surface facing the outside of said particle wherein

a) one or several of the types of subunits have one or several first binding moieties per type of subunit with the binding moiety facing the outside of the particle for binding of any specific ligand binding protein; and

b) i) the particle contains within its shell a marker and/or

ii) one or several of the types of subunits have one or several second binding moieties per type of subunit with the binding moiety facing the inside and/or the outside of the particle for binding a marker; and

c) the marker or markers enables detection of the particle.

2. The nanoparticle according to claim 1 wherein the marker is selected from the group consisting of an enzyme, luminescent protein, fluorescent or coloured protein or organic molecule and rare earth metal.

3. The nanoparticle according to claim 2 wherein the marker is a protein, optionally an enzyme, selected from the group consisting of luciferase, GAO and GFP.

4. The nanoparticle according to claim 2 wherein the marker is a lanthanide preferably selected from the group consisting of Tb, Eu, Sm and Dy.

5. The nanoparticle according to claim 1 wherein one or several of the types of subunits have one or several third binding moieties per type of subunit with the binding moiety facing the outside of the particle for binding to a solid support.

6. The nanoparticle according to any of preceding claims wherein the shell of the nanoparticle is a recombinant virus capsid or a self-assembling shell made of vital capsid protein(s).

7. The nanoparticle according to claim 6 wherein the virus is a phage.

8. The nanoparticle according to claim 7 wherein the phage is selected from the group consisting of T4, K3, RB70 and RB15.

9. The nanoparticle according to any of claims 1 to 5 wherein the shell of the nanoparticle is recombinant apoferritin.

10. The nanoparticle according to any of preceding claims wherein a first binding moiety is selected from the group consisting of protein A, protein G, protein L, calmodulin binding peptide (CBP) and biotin carboxyl carrier protein (BCCP).

11. The nanoparticle according to any of claims 1 to 9 wherein a first binding moiety is an antibody against one of members of the group consisting of CRP, ABO blood group antigens and TSH.

12. The nanoparticle according to any of claims 1 to 9 wherein a second binding moiety is a binding moiety selected from the group consisting of protein A, protein G, protein L, calmodulin binding protein (CBP) and biotin carboxyl carrier protein (BCCP).

13. The nanoparticle according to any of claims 1 to 9 wherein a second binding moiety is an antibody against one of the group consisting of CRP, ABO blood group antigens and TSH.

14. The nanoparticle according to any of preceding claims wherein the minimum radius of the nanoparticle is more than 10 nm, preferably more than 40 nm, even more preferably more than 60 nm.

15. The nanoparticle according to any of preceding claims wherein the number of subunits is more than 8, preferably more than 20, more preferably more than 400, most preferably more than 900.

16. Use of a nanoparticle according to any of preceding claims in a bioaffinity assay.

17. Kit for an immunoassay comprising the nanoparticle according to any of claims 1 to 15.

ABSTRACT

Thus this invention relates to a nanoparticle, useful for bioaffinity assays. The nanoparticle has a self-assembling shell built up of several protein and/or peptide subunits, which protein and/or peptide subunits can be of one or several different types, assembled in an organized manner to form the shell having an inner surface facing the inside and an outer surface facing the outside of said particle. One or several of the types of subunits have one or several first binding moieties per type of subunit with the binding moiety facing the outside of the particle for binding of any specific ligand binding protein; and the particle contains within its shell a marker and/or one or several of the types of subunits have one or several second binding moieties per type of subunit with the binding moiety facing the inside and/or the outside of the particle for binding a marker; and the marker or markers enables detection of the particle.

This invention also relates to a bioaffinity assays using the nanoparticle. This invention further relates to a kit for bioaffinity assays comprising the nanoparticle.

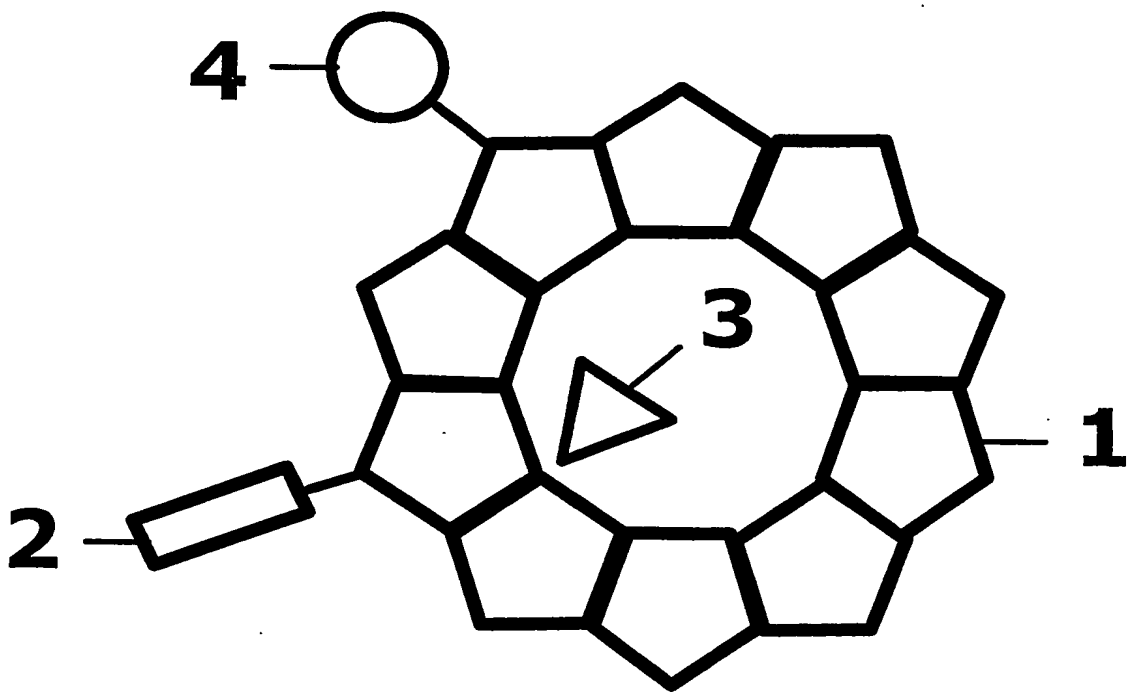


Figure 1

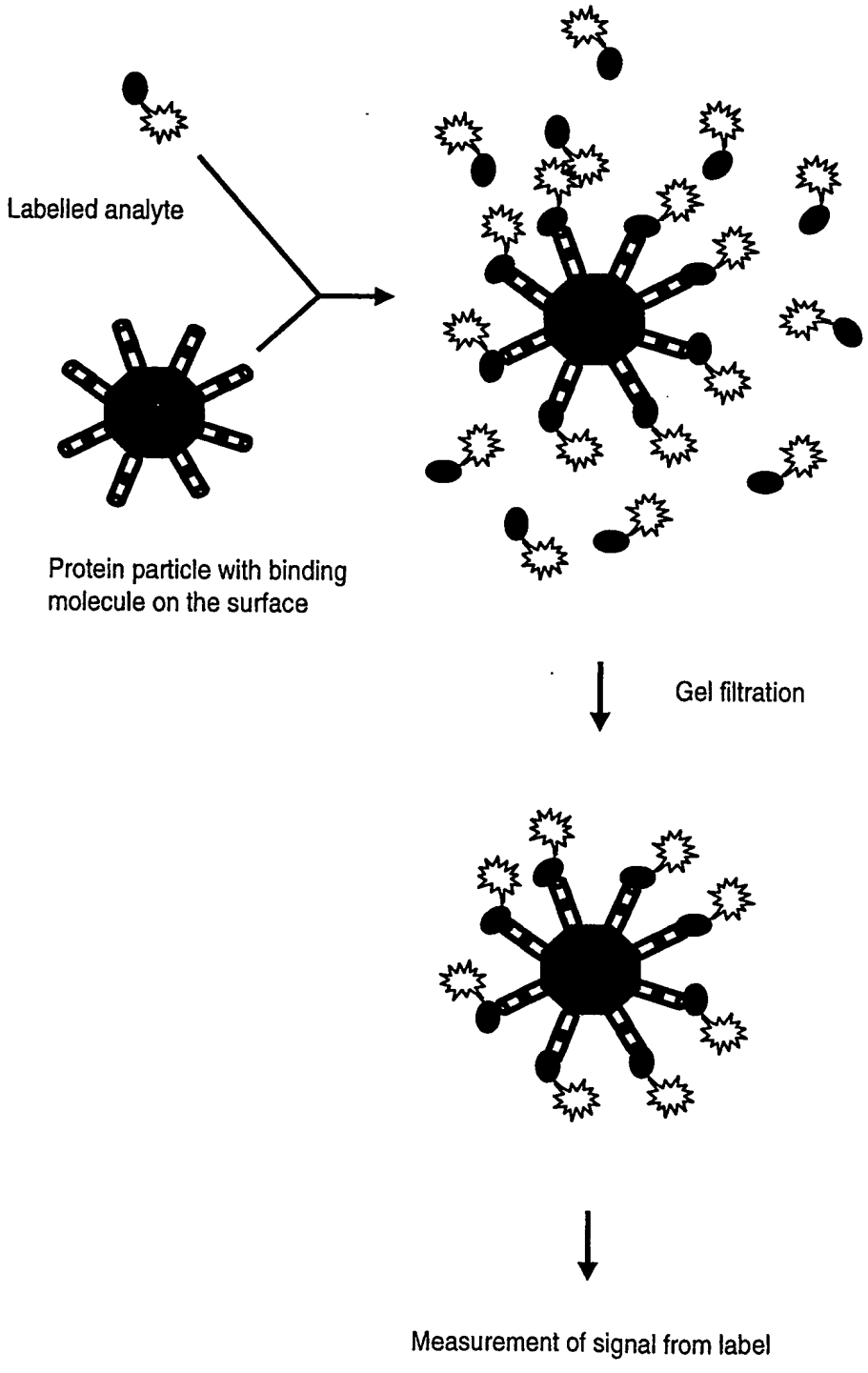
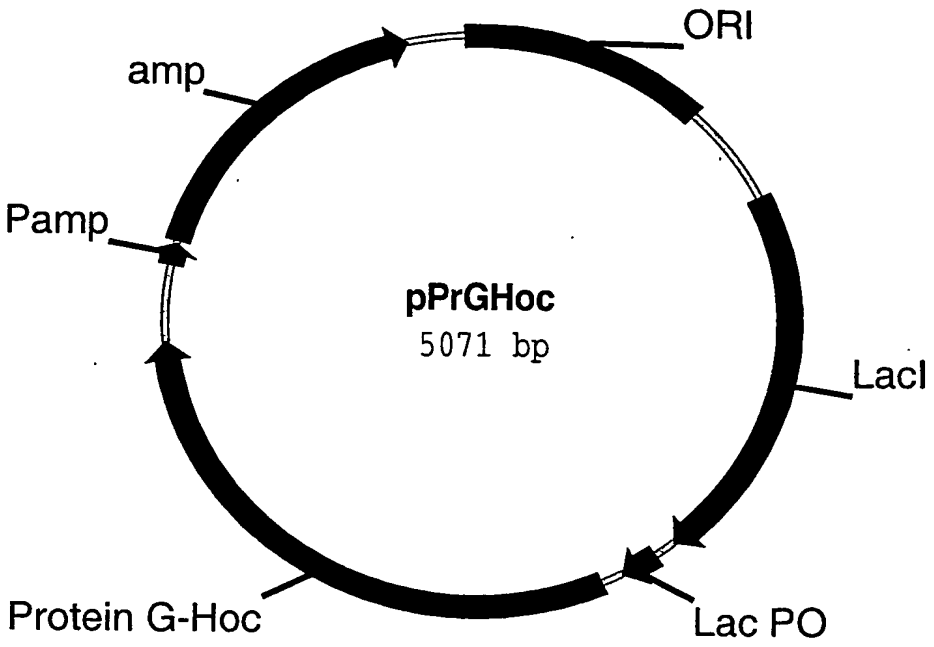

Labelled analyte
Protein particle with binding molecule on the surface
Gel filtration
Measurement of signal from label

Figure 2



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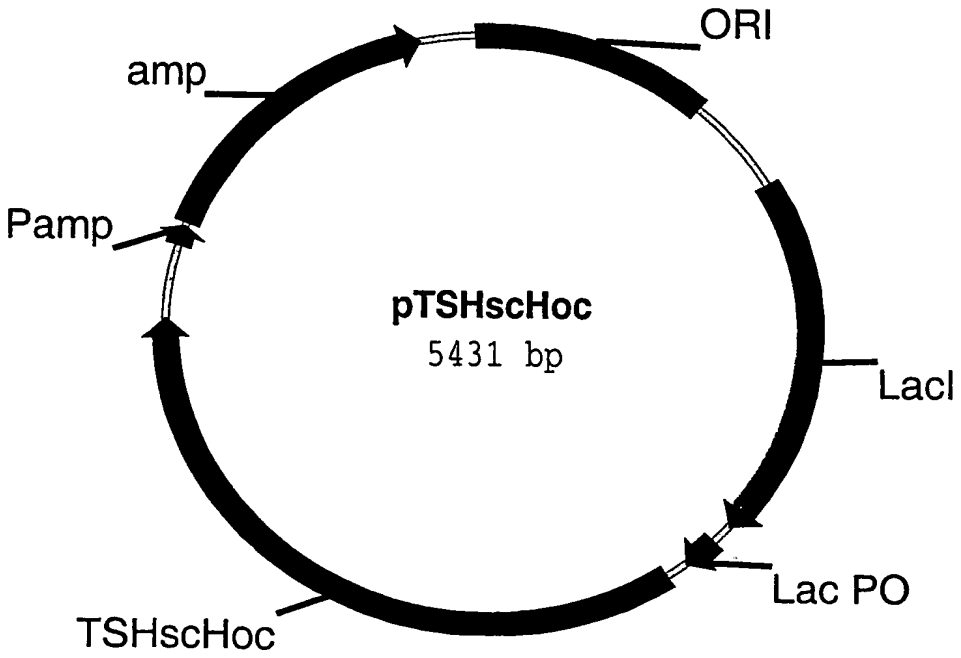

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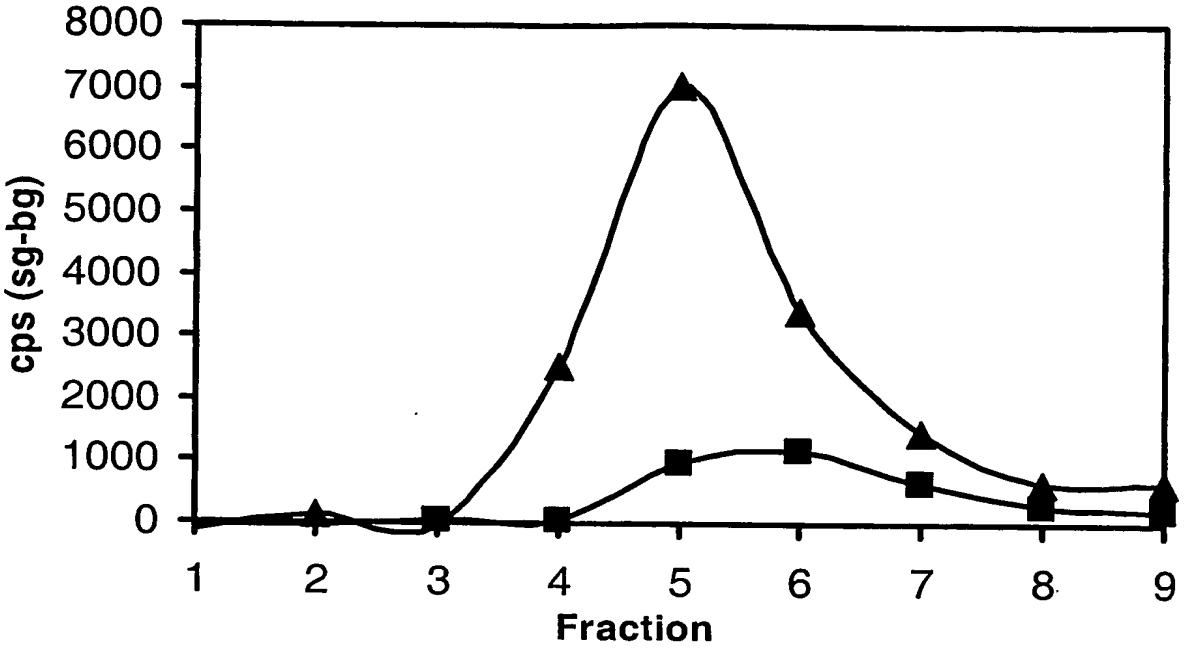

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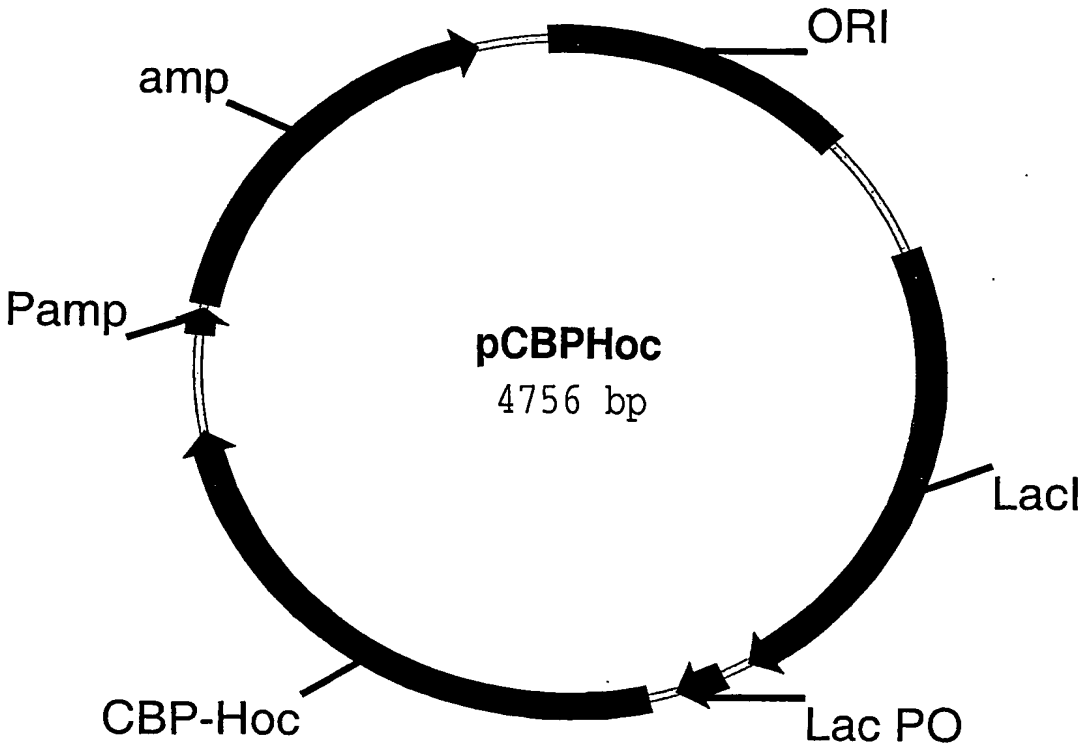

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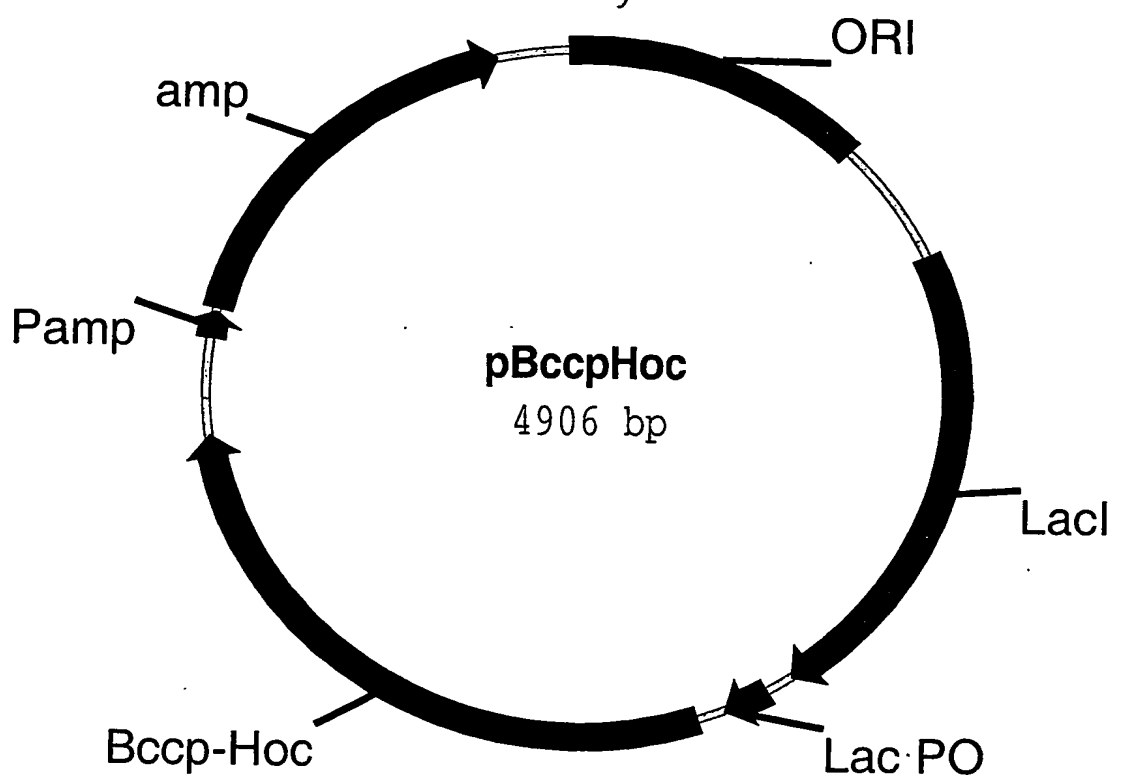

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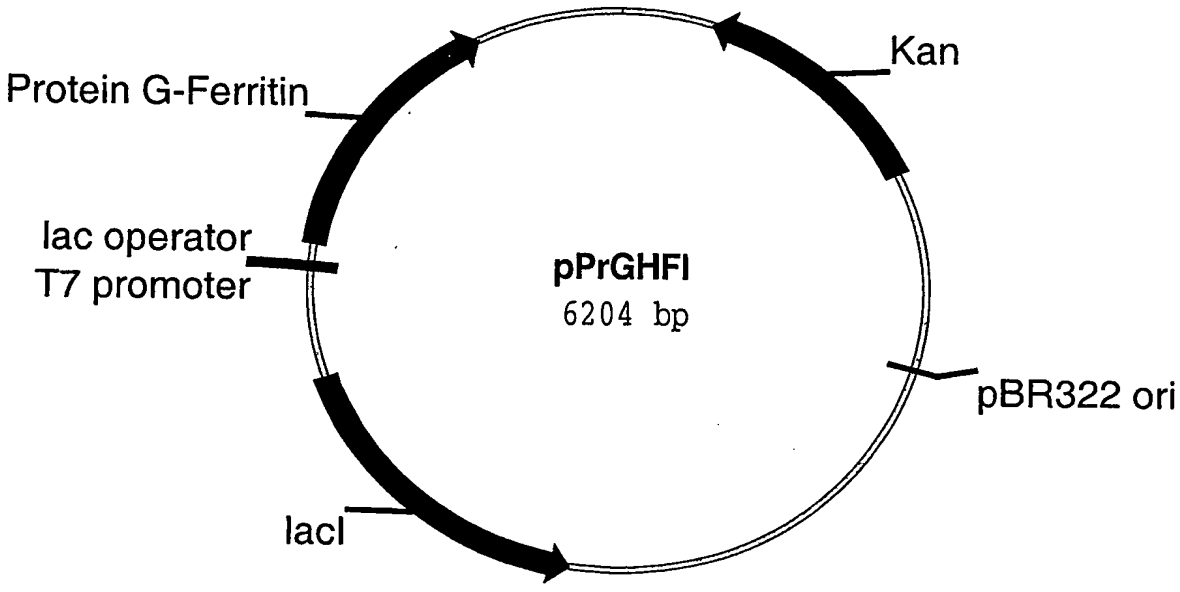

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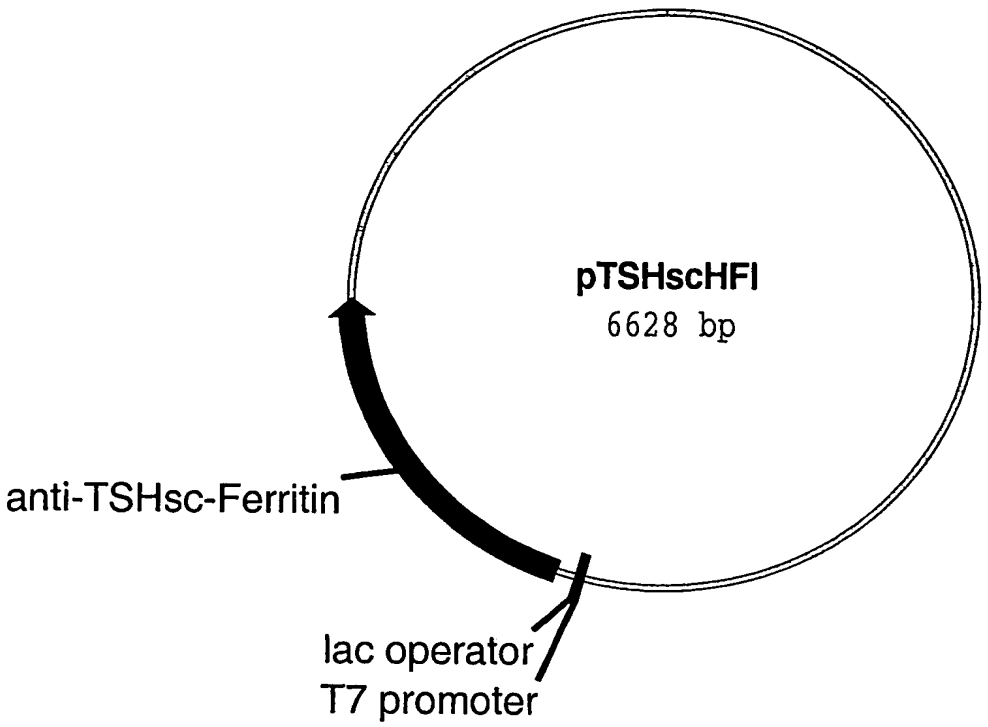

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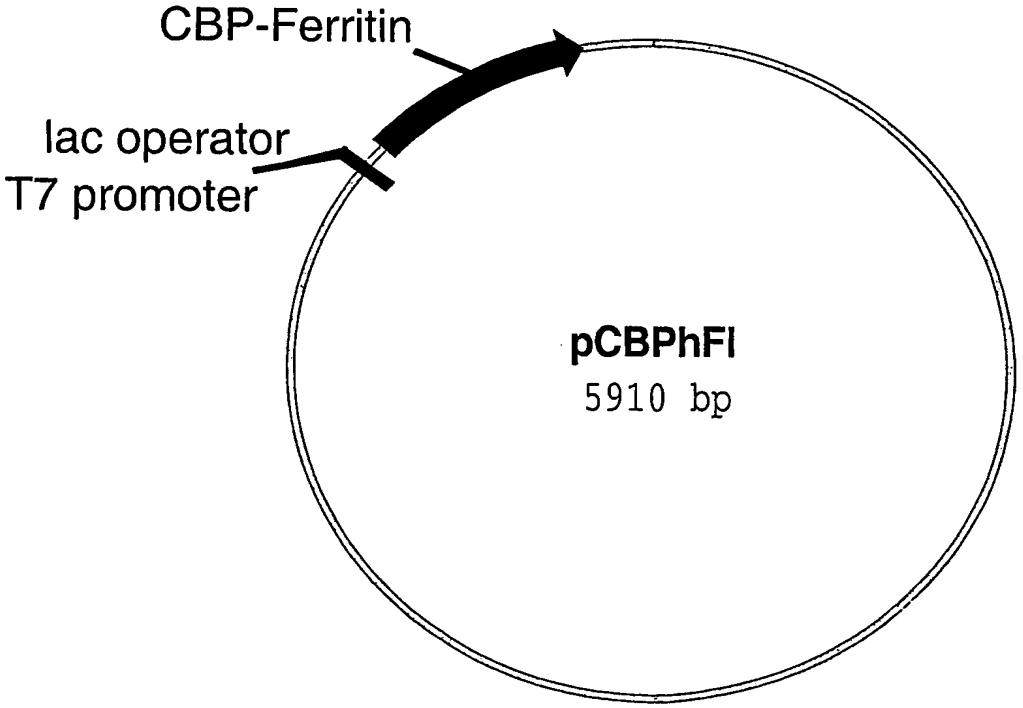

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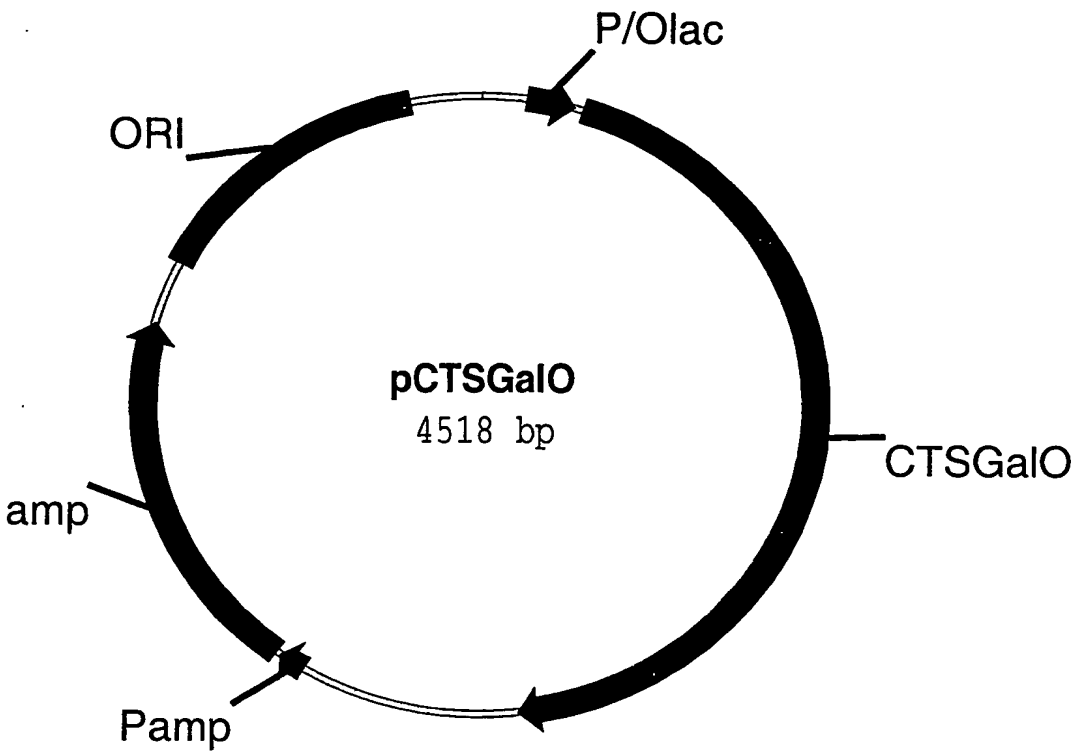

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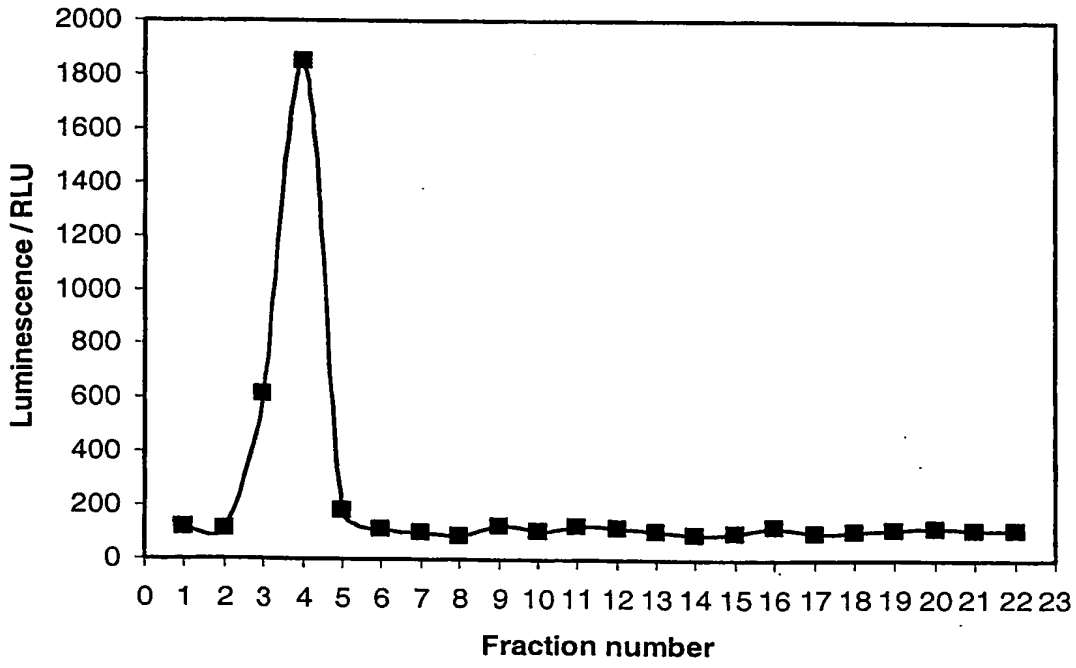

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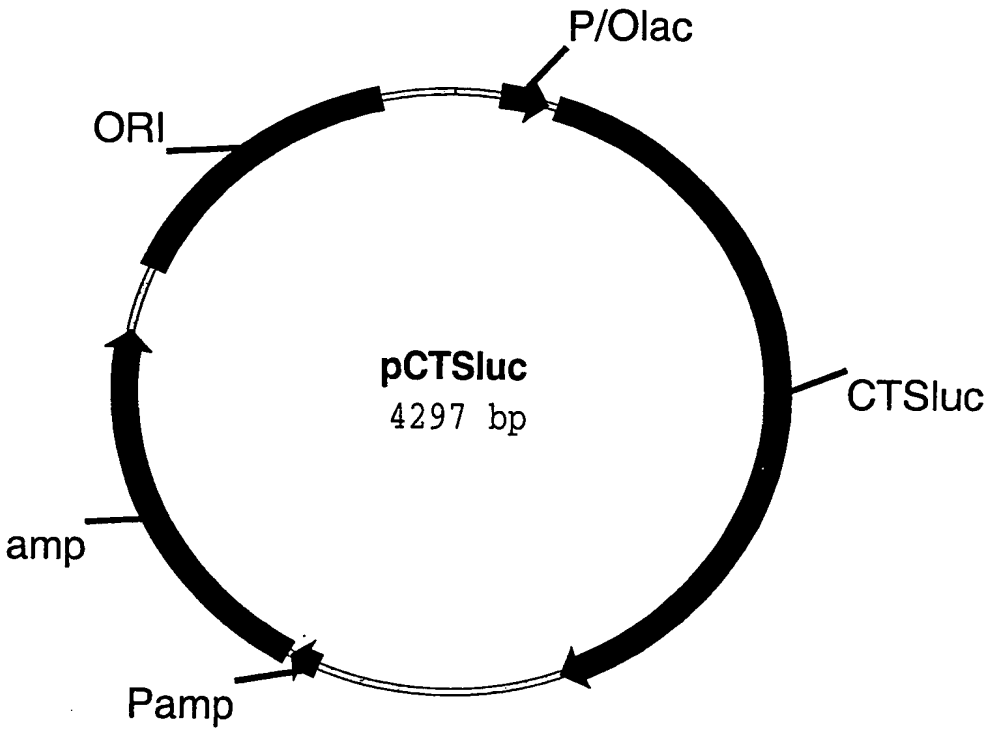

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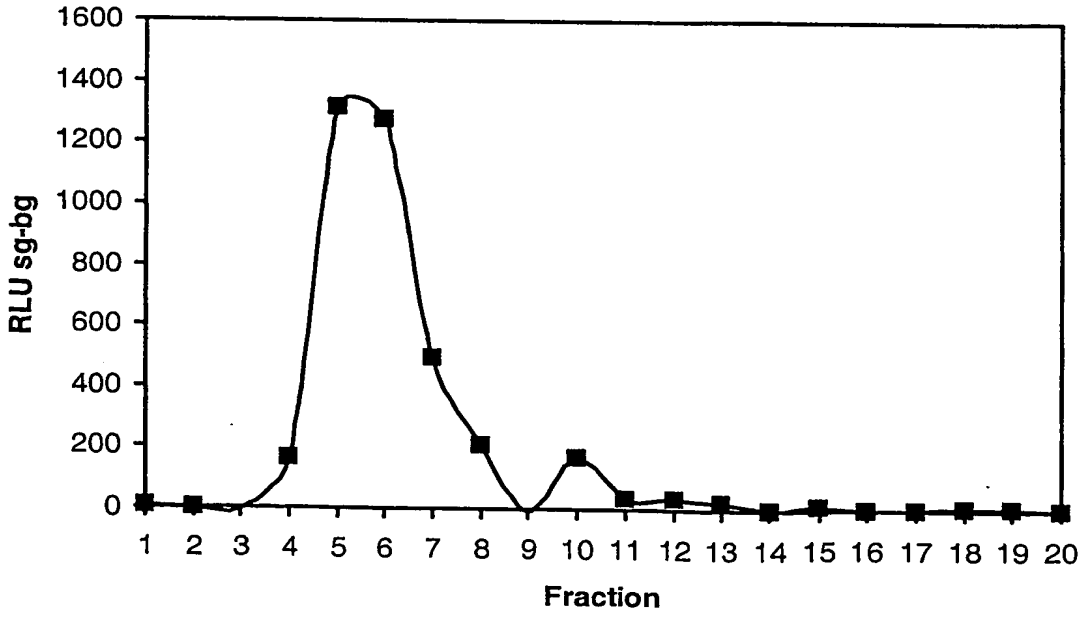

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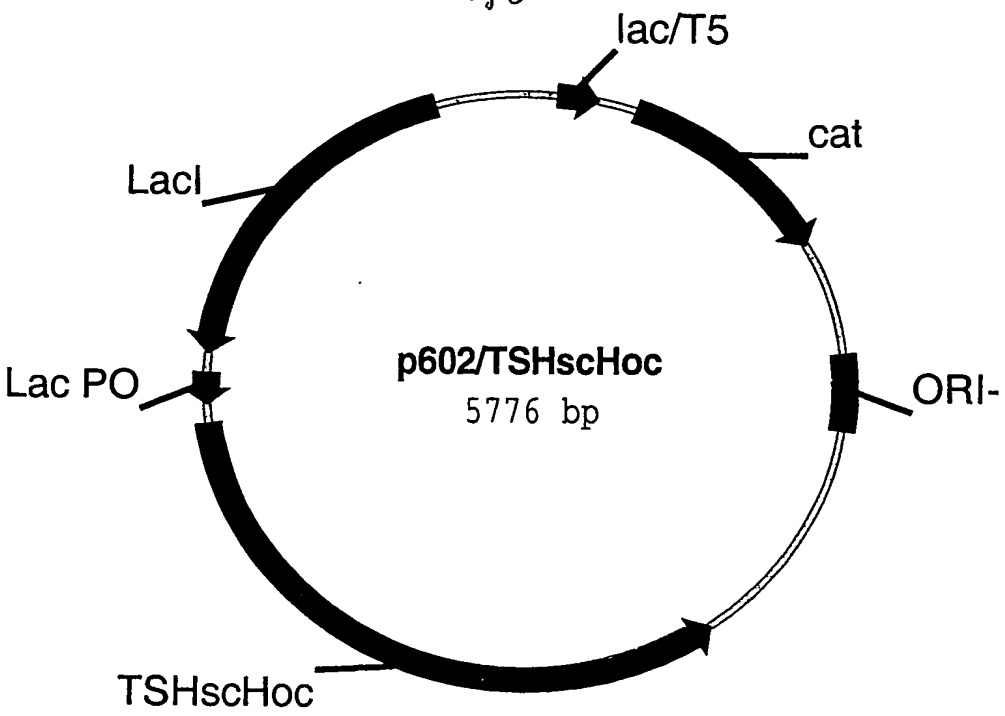

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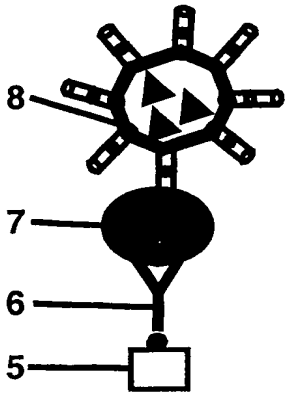

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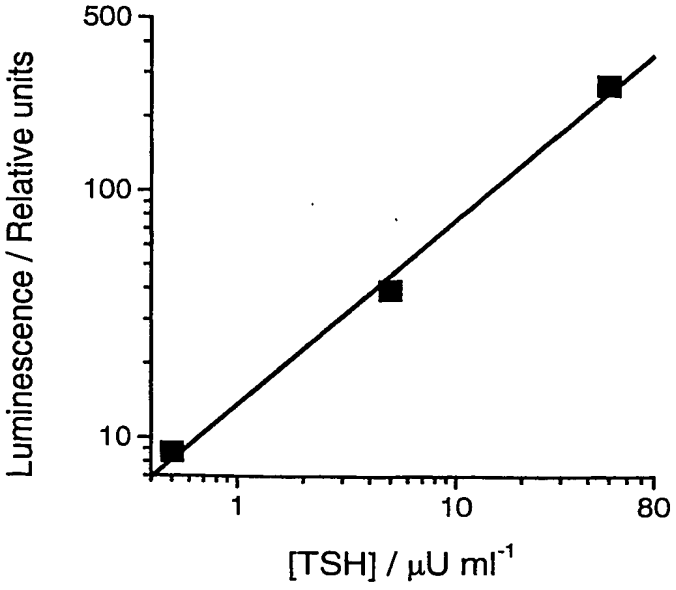

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